● RESEARCH ARTICLE

A rapid absorbance-based growth assay to screen the toxicity of oligomer Aβ$_{42}$ and protect against cell death in yeast

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Abstract

Multiple lines of evidence show that soluble oligomer forms of amyloid β protein (Aβ$_{42}$) are the most neurotoxic species in the brain and correlates with the degree of neuronal loss and cognitive deficit in Alzheimer's disease. Although many studies have used mammalian cells to investigate oligomer Aβ$_{42}$ toxicity, the use of more simple eukaryotic cellular systems offers advantages for large-scale screening studies. We have previously established and validated budding yeast, *Saccharomyces cerevisiae* to be a simple and a robust model to study the toxicity of Aβ. Using colony counting based methods, oligomeric Aβ$_{42}$ was shown to induce dose-dependent cell death in yeast. We have adapted this method for high throughput screening by developing an absorbance-based growth assay. We further validated the assay with treatments previously shown to protect oligomer Aβ$_{42}$ induced cell death in mammalian and yeast cells. This assay offers a platform for studying underlying mechanisms of oligomer Aβ$_{42}$ induced cell death using gene deletion/overexpression libraries and developing novel agents that alleviate Aβ$_{42}$ induced cell death.

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Introduction

Alzheimer's disease is an incurable neurodegenerative disorder featuring progressive loss of memory and ability to perform daily tasks ultimately leading to death. The disease is characterized by deposition of intracellular neurofibrillary tangles and extracellular amyloid plaques within the brain. The cerebral amyloid plaques are insoluble protein deposits associated with dystrophic neurites, inflammatory processes and a dense core comprising primarily of aggregated fibrillar forms of Aβ protein (Fraser et al., 1993). The amyloid β protein (Aβ) is a 40/42-amino acid product of amyloid precursor protein and is found in the brains and cerebrospinal fluid of both healthy individuals and people with AD (reviewed in (Selkoe and Hardy, 2016)). Numerous isoforms of Aβ have been detected in the brain, however the AD brain exhibits markedly increased levels of the longer aggregate-prone isoform, Aβ$_{42}$ [reviewed in Bharadwaj et al. (2009)]. Soluble oligomeric form of Aβ$_{42}$ correlates with cognitive loss in AD as compared to larger fibrillar Aβ aggregates and is considered the most neurotoxic species (McLean et al., 1999; Dahlgren et al., 2002).

In vitro and *in vivo* studies have shown that Aβ oligomers induce neuronal synaptic loss and degeneration and also reduce viability of non-neuronal mammalian cells and non-mamma-

lian cells, including yeast (Bharadwaj et al., 2008; Treusch et al., 2011), suggesting a common mechanism for oligomer Aβ$_{42}$ induced toxicity shared between different cell types. Yeasts are eukaryotic cells and offer greater advantage over other disease models mainly due to the ease of manipulation, availability of powerful genomic and proteomic tools and high-throughput screening techniques. In addition to genetic similarity, yeast cells show similarity in numerous metabolic and signaling pathways with humans, especially those involved in ageing and cellular death. Yeast models have been used extensively to study misfolded or aggregated proteins involved in late-onset neurodegenerative disorders such as Huntington polyglutamine repeats, α-synuclein, superoxide dismutase 1 and Aβ$_{42}$ (Bharadwaj et al., 2010). Due to their robust nature and ability to effectively coordinate the activities of protective stress response pathways to toxic proteins, they are ideally suited for investigating disease relevant pathways and for testing of small molecules that protect against cell death.

Similar to neuronal cells (Dahlgren et al., 2002), we have previously shown that oligomer Aβ$_{42}$ is significantly more toxic than fibrillar Aβ$_{42}$ and induced dose-dependent cell death in yeast (Bharadwaj et al., 2008). This yeast model for Aβ$_{42}$ toxicity have been implemented for investigating the neuroprotective effects of whey derived peptides (Bharad-

waj et al., 2013), functional characterisation of Aβ fusion proteins (Caine et al., 2011) and in studying the role of autophagy in protection against Aβ42 toxicity (Bharadwaj et al., 2012). These assays have been based on colony counting which is time-consuming to set-up and unsuitable for screening of gene deletion/overexpression libraries and small molecule collections. To increase feasibility for such broad-based screening methods, here we have developed a growth based high-throughput assay based on our established colony-count method to measure oligomer Aβ42 induced cell death. This novel assay was further validated by assessing known protective agents that promote survival against oligomer Aβ42 mediated cell death.

Materials and Methods

Yeast strain
Wild-type *Saccharomyces cerevisiae* strain BY4743 [genotype: *MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*] (Dr. Gabriel Perrone University of New South Wales) was used for this study. Yeast cells were grown on YEPD (1% yeast extract; 2% peptone; 2% dextrose), YNB (yeast nutrient base) synthetic complete or nitrogen starvation media, YNB (-N) for toxicity experiments. Yeast cells were stored as glycerol stocks at –80°C and in media plates at 4°C.

Reagents
Autophagy modulating compounds including Latrepirdine, Rapamycin and SMER28 were used as controls in this study. Latrepirdine (Dimebolin dihydrochloride, molecular weight (MW): 392.37) was purchased from Biotrend AG, Zurich, Switzerland. Latrepirdine was dissolved into sterile double distilled water to 30 mM concentration. Rapamycin and SMER28 were purchased from Sigma (Missouri, USA) and solutions were prepared in DMSO (Dimethyl sulfoxide). Freshly prepared working stocks (1 mM) prepared from -80°C frozen stocks were used for all experiments. Synthetic human Aβ42 and Aβ16 were purchased from ERI Laboratory (Connecticut). Recombinant human insulin (I2643, MW: 5800 Da) was purchased from Sigma.

Aβ preparation
Aβ peptides were prepared according to Bharadwaj et al. (2012). Aβ peptide (0.5 mg) was dissolved in 500 μL hexafluoroisopropanol solution and incubated overnight at room temperature. The hexafluoroisopropanol was then evaporated using vacuum and the dried peptide films were stored in a –80°C freezer. Aβ peptide films were dissolved in 200 μL sterile double-distilled water and prepared prior to use. The peptide solution was vortexed followed by sonication for 5 minutes on ice and then centrifuged for 10 minutes at 14,000 × *g*. The resulting supernatant containing soluble Aβ oligomers and was used for yeast toxicity assays following overnight incubation at room temperature to allow oligomerisation. Insulin (MW: 4.8 kDa) was freshly prepared in 1 × PBS and truncated Aβ16 (MW: 1.6 kDa) was prepared in a similar fashion as Aβ42 for toxicity assays.

Aβ toxicity assay in yeast: colony forming unit assay
Wild-type *Saccharomyces cerevisiae* (strain, BY4743) was used for Aβ toxicity experiments. Yeast cells were stored on YEPD agar plates at 4°C. A single colony was inoculated in YEPD liquid media and incubated by shaking at 30°C overnight. The overnight yeast culture was re-inoculated in fresh YEPD liquid media and incubated with shaking and grown for 3–4 hours at 30°C to reach exponential phase growth. For inducing autophagy, cells were washed in sterile water and pre-incubated with latrepirdine (5, 10, and 20 μM), rapamycin (50, 100, 150, and 200 nM), SMER28 (small molecule enhancer of rapamycin, 10, 20, and 50 μM) or incubated in nitrogen starvation media (YNB, -N). These cultures were diluted to ~5 × 10^3 yeast cells/mL in sterile distilled water. Cells were then dispensed into 96-well microtitre plates for peptide treatments. Vehicle or peptide preparations were added to the diluted cell suspension to required concentrations. The final volume in each well was made up to ~100 μL and then the microtitre plate was sealed with a gas permeable membrane (optional) and incubated at 30°C by shaking for time periods as indicated. The cell suspensions were then plated on YEPD agar plates and incubated at 30°C for 2 days to measure the number of colony-forming units (CFU). Cell viability following peptide treatment was calculated from the CFU count and represented as percent change compared to vehicle treatment.

Absorbance-based growth assay
In this study, we adapted the colony forming unit assay to an absorbance-based growth assay using a 96-well plate format to increase the feasibility of this yeast model in large scale screening techniques. Cells were treated with peptides and/or autophagy modulating reagents as described above. Exponentially growing yeast cells were treated with increasing doses of oligomeric Aβ42 for 24 hours, and then the cell suspensions were supplemented with yeast growth media (YEPD, 50 μL) and the growth was measured after 16-hour incubation at 30°C by absorbance measurement (optical density, OD600 nm) using a spectrophotometer FLUOstar Optima plate reader (BMG Labtech, Ortenberg, Germany). The cell density measured as absorbance values was represented as percent cell viability. Cell viability in peptide treated samples was evaluated from the absorbance values from similar time points as the untreated and represented as percent change from vehicle treated.

Statistical analysis
For the colony count assay cell viability was determined by counting colonies across the various treatments as described previously (Bharadwaj et al., 2008). For the growth assay, the mean absorbance values between treatment replicates were used to determine cell survival across the various peptide treatments used. All comparisons were made using two-tailed Student's *t*-test (equal sample size, unequal variances) using GraphPad Prism software version 8 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Oligomer Aβ$_{42}$ toxicity

Initially, we compared the colony forming unit assay and the absorbance-based growth assay to measure oligomer Aβ$_{42}$ toxicity (**Figure 1**). Both assays revealed a dose dependent toxicity profile, however colony counting showed greater sensitivity at lower Aβ$_{42}$ concentrations (0.5–2 μM) (**Figure 1B**). Both assays were comparable at higher Aβ$_{42}$ concentrations (4–10 μM) (**Figure 1B**). We also compared the toxicity of oligomer Aβ$_{42}$ as the positive control and non-aggregating C-terminal truncated Aβ$_{16}$ peptide (MW 1760 Da) and insulin peptide (MW 5800 Da) were used as the negative controls (**Figure 2**). Oligomer Aβ$_{42}$ showed a dose-dependent loss in cell viability, whereas Aβ$_{16}$ and insulin treatment did not alter cell viability at similar concentrations tested (**Figure 2**). It is noted that cell viability analysis using the growth-based assay showed 50–80% survival at 2 μM, 40–60% at 4 μM and 10% at 10 μM in **Figure 1B**, whereas **Figure 2** showed 75–85% survival at 2 μM, 50% at 4 μM and 10% at 10 μM.

Inhibitors of oligomer Aβ$_{42}$ toxicity

Wild type yeast was incubated in nitrogen depleted media (**Figure 3A**) or treated with latrepirdine (5–20 μM) (**Figure 3B**), rapamycin (50–200 nM) (**Figure 3C**), or SMER28 (10–50 μM) to induce autophagy followed by oligomer Aβ$_{42}$ treatment (2–10 μM) (**Figure 3D**). A dose dependent loss in viability was observed in cells treated with oligomeric Aβ$_{42}$. Low concentrations of Aβ$_{42}$ (2 μM) showed ~20% cell death and higher concentrations ranging 4–10 μM showed 50–80% loss in viability. Cell viability following Aβ$_{42}$ treatment was significantly higher in nitrogen starved, latrepirdine, rapamycin or SMER28 treated cells compared to vehicle treated (**Figure 3**). Notably, only nitrogen starvation showed significant protection (a 40% increase in viability) against the highest Aβ$_{42}$ concentration tested (10 μM). Whereas, latrepirdine, rapamycin and SMER28 increased cell survival (20–60% increase in viability) only at lower Aβ$_{42}$ concentrations (2–5 μM). The incubation period with the autophagy enhancers was found to be critical for inducing protection against oligomer Aβ$_{42}$ toxicity. Shorter incubation times (6 hours, 30°C) were used for latrepirdine, rapamycin and nitrogen starvation. However, longer incubations (16 hours, 30°C) were required for SMER28 to promote cell viability with Aβ$_{42}$ treatment (**Figure 3**). The results showed significant differences in the levels of protection against oligomer Aβ$_{42}$ toxicity conferred by physiological (nitrogen starvation) and chemical inducers (latrepirdine, rapamycin and SMER28) of autophagy.

Discussion

A novel high-throughput assay for measuring oligomer Aβ$_{42}$ toxicity

Cell based high-throughput assays are indispensable for analysis of signaling pathways and discovery of small-molecule modulators of disease processes. The budding yeast, *Saccharomyces cerevisiae* has been broadly employed for mechanism-of-action studies and drug target identification.

Existing high-throughput viability assays such as colony count and spotting assay are based on clonogenic analysis (Bharadwaj et al., 2010), which measures the effectiveness of specific agents on the survival and proliferation of yeast cells in agar media. Compared to such clonogenic assays, absorbance-based viability assays offer a simple and more sensitive technique for screening gene deletion/overexpression libraries and compound repositories.

In our previous work, we developed a novel assay for measuring toxicity of oligomer Aβ$_{42}$ peptide, a key protein associated with pathogenesis and disease development in AD (Bharadwaj et al., 2008, 2012). Similar to neuronal cells, oligomer Aβ$_{42}$ was found to be more toxic and induced dose dependent cell death in yeast compared to fibrillar Aβ$_{42}$. The original method used a colony count based assay to measure oligomer Aβ$_{42}$ toxicity (Bharadwaj et al., 2008). In this study, we have developed and validated an absorbance based high-throughput assay to measure toxicity of oligomer Aβ$_{42}$. It is noted that cell viability analysis using the growth-based assay showed slight variability in **Figures 1B** and **2**. It is likely that the differences in the standard deviation is due to the number of replicates conducted for experiments in **Figures 1B** ($n = 8$) and **2** ($n = 12$). However, the overall level of toxicity observed in **Figures 1B** and **2** are consistent. Notably, both the colony count and the absorbance-based growth assay in yeast showed significant loss in cell viability at oligomer Aβ$_{42}$ concentrations (≥ 1 μM) required to induce toxicity in neuronal cells (Dahlgren et al., 2002). This yeast assay offers a robust and high-throughput cell-based platform for analysis of disease relevant pathways and screening compounds that can alleviate oligomer Aβ$_{42}$ induced cell death.

Previous studies have developed high-throughput yeast assays to measure Aβ$_{42}$ induced toxicity by targeting it to the secretory pathway (Treusch et al., 2011) or used strains expressing Aβ$_{42}$ fused to the essential functional domain of Sup35, a prion protein, to measure Aβ$_{42}$ oligomerization (Park et al., 2011). Some of the advantages of our yeast model compared to the studies of Park et al. and Treusch et al. include 1) ability to test toxicity of different Aβ peptides and its various isoforms and 2) suitability for genetic screening as the assay uses structurally characterized synthetic Aβ$_{42}$ preparations and eliminate the effects of gene deletion/overexpression on Aβ$_{42}$ production. Also, the use of an absorbance-based growth assay increases its suitability for screening gene deletion/overexpression and drug libraries as compared to the use of fluorescent/colorimetric dyes which has limitations due to likelihood of toxicity in certain mutants, interference with cell metabolism and fluorescent/colorimetric assay read out. Overall, data from the absorbance-based assay indicated its suitability for large scale, high-throughput screening methods and offers a robust cell-based platform for analysis of disease relevant pathways and screening compounds that can alleviate oligomer Aβ$_{42}$ induced cell death.

Induction of autophagy protects against oligomer Aβ$_{42}$ toxicity

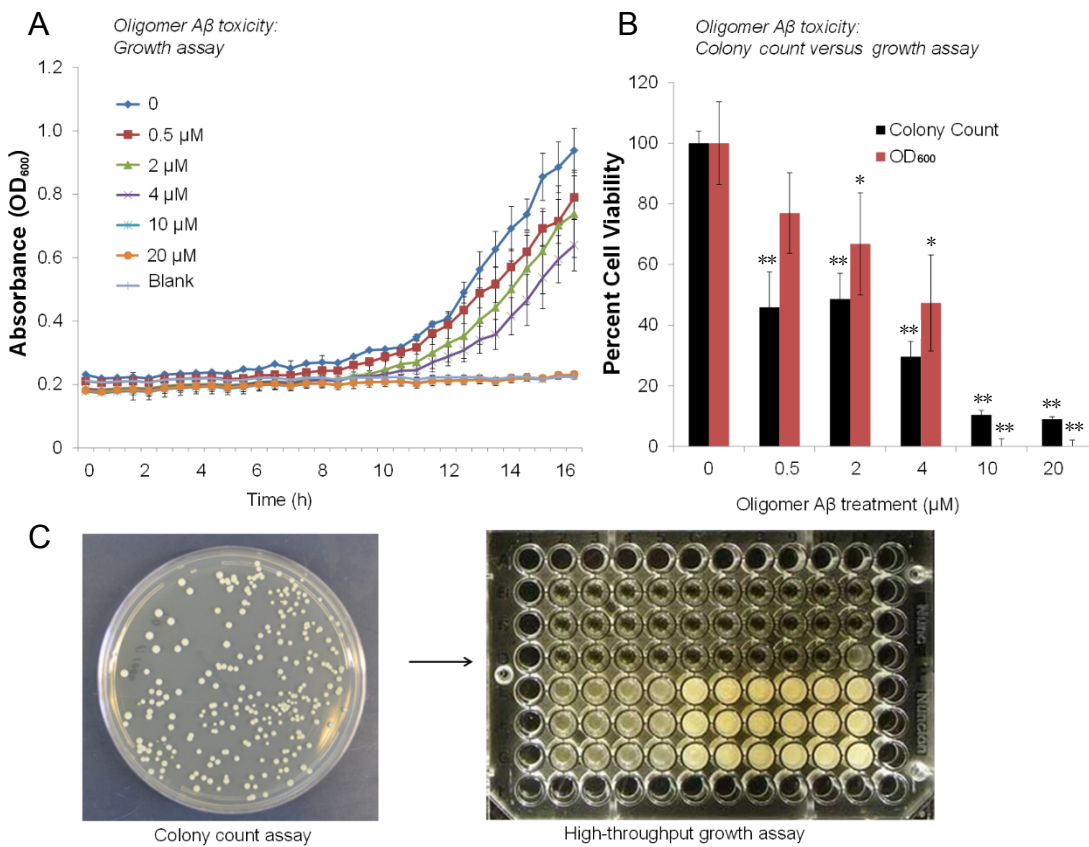A wide range of inhibitors of Aβ$_{42}$ aggregation, including

Figure 1 Comparative analysis of oligomer Aβ42 toxicity in yeast by colony count and the absorbance-based high-throughput assay.
(A) Exponentially growing yeast cells in YEPD (1% yeast extract; 2% peptone; 2% dextrose) media were serially diluted in sterile double distilled water and treated with oligomer Aβ42 peptide for 24 hours. Following treatment, the cell suspensions were supplemented with fresh YEPD media and the growth/cell density was monitored for 16 hours at 30°C by absorbance measurement (optical density, OD_{600}). (B) The absorbance values measured at 16 hours are represented as percent cell viability for each treatment and directly compared to those values obtained with the established colony count assay (Bharadwaj et al., 2008). Although less sensitive at low concentrations (1–2 μM) of oligomer Aβ treatment compared to the colony count assay, the high-throughput assay shows dose-dependent loss of viability with oligomer Aβ42 treatment, compared to vehicle treatment. Statistical significance between treatments was assessed using two-tailed Student's t-test ($n = 8$, Aβ42 treated *vs.* untreated, ∗$P < 0.05$, ∗∗$P < 0.01$). (C) Images showing the colony count and the high-throughput assay. Aβ: Amyloid β protein.
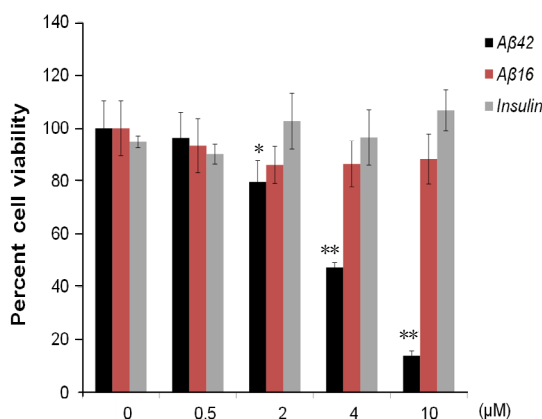

Figure 2 Toxicity of oligomer Aβ42, Aβ16 and insulin peptides.
Exponentially growing yeast cells in YEPD (1% yeast extract; 2% peptone; 2% dextrose) media were serially diluted in sterile double distilled water and treated with oligomer Aβ42, Aβ16 and insulin peptide preparations for 24 hours. Oligomer Aβ42 treatment showed dose dependent loss of viability, whereas Aβ16 and insulin treatment were significantly less toxic as compared to vehicle treated. Statistical significance between treatments was assessed using two-tailed Student's t-test ($n = 12$, Aβ42 treated *vs.* untreated, ∗$P < 0.05$, ∗∗$P < 0.01$). Aβ: Amyloid β protein.

peptide and small molecules of natural and synthetic origin have been shown to regulate toxicity of Aβ42 (Mangialasche et al., 2010). An important caveat in designing such inhibitors is to target the toxic form of Aβ42 and identify compounds with low cytotoxicity. In addition to inhibiting the formation or disaggregating the Aβ42 toxic oligomer structures, there is growing interest towards inducing effective cellular responses to Aβ42 induced toxicity and cell death, and thereby suppress the downstream apoptotic cascades and necrosis in the AD brain. A cellular pathway that can confer such protection is autophagy (Nixon, 2013). Autophagy is a vital intracellular catabolic pathway that plays an important role in the removal of aggregated or misfolded proteins in neurodegenerative diseases. Modulation of autophagy can enhance protective responses to toxicity, and suppress apoptotic cascades or necrosis leading to neurodegeneration in the brain (Mputhia et al., 2019).

Substantial benefits have been observed with autophagy-inducing agents in various neurodegenerative disease models (Nixon, 2013). A widely used inducer of autophagy, rapamycin is a selective inhibitor of mTOR (mammalian tar-
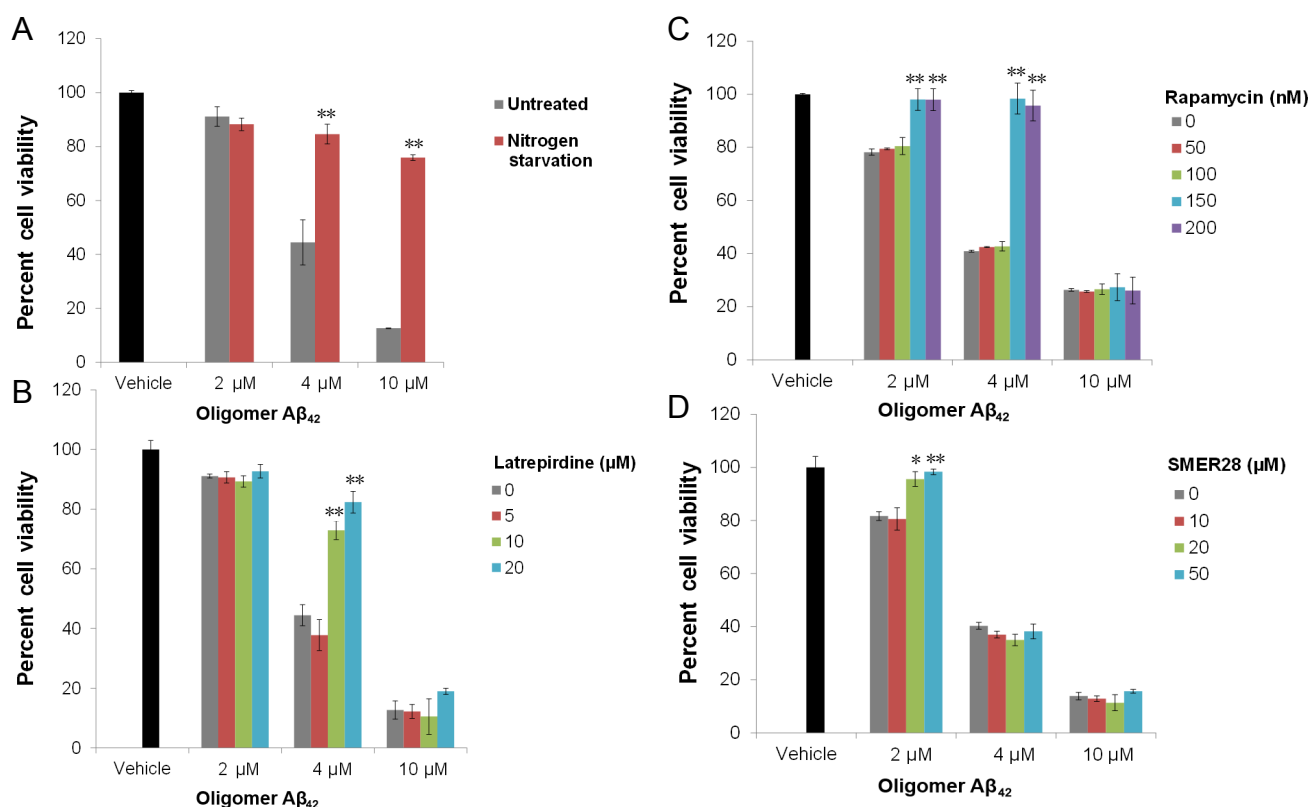
Figure 3 Induction of autophagy protects against oligomer Aβ₄₂ toxicity in yeast.
Exponentially growing yeast cells were incubated in (A) nitrogen depleted media or (B) YNB (yeast nutrient base)-synthetic complete media containing autophagy modulating compounds, i.e., latrepirdine (5, 10 and 20 μM) or (C) rapamycin (50, 100, 150 and 200 nM) and (D) SMER28 (10, 20 and 50 μM)) at 30°C to induce autophagy followed by oligomer Aβ treatment. Loss of cell viability from the different treatments was then measured using the absorbance-based Aβ₄₂ toxicity assay. Cell viability following oligomer Aβ₄₂ treatment was significantly higher in nitrogen starved or latrepirdine treated wild type cells compared to vehicle treated. Statistical significance between treatments was assessed using two-tailed Student's *t*-test (Different Aβ₄₂ treated groups were compared. In the Aβ₄₂ treated groups, control cells and cells treated with autophagy modulators were compared. *$P < 0.05$, **$P < 0.01$). Only nitrogen starvation showed significant protection (40% increase in viability) against 10 μM Aβ₄₂. Latrepirdine, rapamycin and SMER28 increased cell survival (20–40% increase in viability) only at 2–5 μM Aβ₄₂. Aβ: Amyloid β protein.

get of rapamycin) (Huang et al., 2004). Rapamycin has been shown to reduce Aβ induced neurodegeneration and improve cognition in transgenic mouse models (Spilman et al., 2010). An mTOR-independent autophagy inducer, SMER28 (small-molecule enhancer of rapamycin) (Huang et al., 2004) has shown to reduce the levels of Aβ and alleviate toxicity in Huntington disease model (Tian et al., 2011). Recently, we showed that enhanced autophagic activity mediated by rapamycin and a pro-neurogenic compound, latrepirdine protected yeast cells against oligomer Aβ₄₂ toxicity (Bharadwaj et al., 2012). We also showed that latrepirdine enhanced mTOR- and Atg5-dependent autophagy and improved cognition in AD mouse model (Steele et al., 2012). Here, we validated the use of the absorbance-based Aβ₄₂ toxicity assay by determining the protective ability of different classes of autophagy enhancers. The results showed significant differences in the levels of protection against oligomer Aβ₄₂ toxicity conferred by physiological (nitrogen starvation) and chemical inducers (latrepirdine, rapamycin and SMER28) of autophagy. These findings indicate how autophagy can be modulated distinctly by different cellular pathways of induction and how they respond to increasing levels of toxicity and cellular damage caused by oligomeric Aβ₄₂.

Previous studies favour a strategy of enhancing the efficacy of autophagy by preventing or reversing the impairments of the specific processes that are disrupted. Several compounds with autophagy-related functions as the primary or secondary drug target have been trialled for several neurodegenerative diseases including AD (Nixon, 2013). Although autophagy activation is a promising intervention, there are some caveats to consider (Sarkar, 2013; Mputhia et al., 2019). In diseases like AD, where there is a pre-existing impairment in autophagy, the success of any autophagy-based intervention may depend on whether lysosomal clearance is functional (Nixon, 2013; Mputhia et al., 2019; Tripathi et al., 2019). In addition, effects of autophagy activation may also vary significantly depending on the physiological state of the cell, especially during proteotoxic stress. In fact, some studies suggest that autophagy activation may be harmful in ageing conditions with pre-existing pathology (Carosi and Sargeant, 2019; Tripathi et al., 2019).

In summary, our previous work (Bharadwaj et al., 2012) together with the current findings provides evidence for autophagy as a preventative treatment against oligomer Aβ₄₂ mediated cell death. In addition, here we have established the potential use of the high-throughput yeast assay as a bi-

ological screen to dissect disease-relevant pathways and network of genes that mediate protection against Aβ42-induced cell death. Although yeast is an excellent screening model for inhibitors of Aβ42 toxicity, it has limitations due to lack of specialized neuronal structures and functions. However, such cell-based systems are necessary for drug discovery and genetic screening, where large, diverse small molecule collections can be tested for their therapeutic efficacy in AD. Such biological assays can cast a much wider net in the discovery of potential leads and even identify undesirable targets of drugs as compared to target-by-target approaches.

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